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Duplication of the Structural Gene for Glycyl-transfer RNA Synthetase in *Escherichia coli*

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Earlier, Folk & Berg (1970*a*,*b*) described a class of mutants affecting the structural gene for glycyl-transfer RNA synthetase (*glyS*); their Gly$^-$ phenotype (requirement for added glycine to minimal medium) was thought to result from a lowered affinity of the enzyme for glycine and consequently a reduced rate of esterification of glycine to tRNA^{Gly} at normal intracellular levels of glycine. Such mutants revert to a Gly$^+$ phenotype at a high frequency ($> 10^{-5}$) although the *glyS* mutant allele remains unchanged.

Our experiments show that the Gly$^+$ revertants arise because of a duplication (or perhaps an amplification) of the mutant *glyS* allele. The duplication can also include other genes which occur up to four minutes map distance from *glyS*. We surmise that the increase in the number of structural genes for glycyl-tRNA synthetase generates an elevated level of the defective enzyme *in vivo* thereby permitting nearly normal rates of esterification of tRNA^{Gly} even with the otherwise limiting levels of endogenous glycine.

1. Introduction

Selection for mutations causing gene duplication was first described by Horiuchi, Horiuchi & Novick (1963). By causing *Escherichia coli* to grow in a chemostat in the presence of limiting quantities of lactose they enriched for cells able to hyperproduce β-galactosidase; hyperproducing strains make more β-galactosidase than normal strains because they contain multiple copies of the *lac* genes per genome. Gene duplication can also be detected when recessive lethal mutations are selected for: certain *trpA36* missense suppressor mutations (su_{36}^+) (Hill, Foulds, Soll & Berg, 1969; Carbon, Squires & Hill, 1969) can be recovered only in strains which have also generated duplicate copies of the wild-type, su_{36}^-, gene (Hill, unpublished observations). In this paper we report another instance of gene duplication involving a portion of the *E. coli* chromosome encompassing the structural gene for glycyl-transfer RNA synthetase (*glyS*).

In an earlier report (Folk & Berg, 1970*a*,*b*), we described a class of glycine-requiring (Gly$^-$) mutants of *E. coli*, in which the glycine auxotrophy resulted from a defective glycyl-tRNA synthetase. Their inability to grow in minimal medium was correlated with a failure to esterify tRNA^{Gly} chains in the absence of added glycine; nearly normal levels of intracellular glycyl-tRNA were produced when the growth medium

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was supplemented with glycine. The limited formation of glycyl-tRNA in minimal medium probably stems from the reduced affinity of the mutant enzyme for glycine, since the K_m for the amino acid with the mutant enzymes is about 20 times higher than of the wild-type progenitor.

Reversion from the Gly^- to a Gly^+ phenotype occurred with an unexpectedly high frequency ($>10^{-5}$) (Folk & Berg, 1970*a*). Transductional analysis with phage P1 showed that the revertants still contained the mutant *glyS* allele; although the levels of glycyl-tRNA synthetase activity were two- to fourfold higher in extracts of the revertants compared to the corresponding mutant, the K_m for glycine, and the stability of the revertant and mutant enzymes, were identical.

Our experiments show that the Gly^+ revertants arise because of a duplication (or perhaps an amplification) of the mutant *glyS* allele. The duplication can also include other genes which occur up to four minutes map distance from *glyS*. We surmise that the increase in the number of structural genes for glycyl-tRNA synthetase generates an elevated level of the defective enzyme *in vivo* thereby permitting nearly normal rates of esterification of $tRNA^{Gly}$ even with the otherwise limiting levels of endogenous glycine.

2. Materials and Methods

(a) *Reagents and media*

^{14}C-labeled amino acids were obtained from New England Nuclear Corp. ^{14}C-labeled indole-3-glycerol-phosphate was a gift of Dr Larry Soll. *E. coli* B tRNA was purchased from Schwarz Bioresearch and General Biochemicals. All other reagents were of the highest purity available from commercial sources.

Most of the growth media were as described previously (Folk & Berg, 1970*a*). The MacConkey agar medium was prepared as described by Cozzarelli & Linn (1966).

(b) *Preparation of cell extracts*

A frozen cell pellet was suspended to a concentration of 20% (w/v) in 0·1 M-potassium phosphate buffer (pH 7·0) with 0·01 M-β-mercaptoethanol. Acid-washed glass beads were added and the suspension was treated for 90 sec with a Mullard sonicator. The extracts were clarified by centrifugation at about 12,000 *g* for 10 to 15 min. Extracts normally contained between 15 and 25 mg of protein/ml., as determined by the method of Lowry, Rosebrough, Farr & Randall (1951) with crystalline bovine plasma albumin as a standard.

(c) *Enzyme assays*

Glycyl-tRNA synthetase activity was assayed by measuring the rate of formation of [^{14}C]glycyl-tRNA by the procedure of Calendar & Berg (1966). The reaction mixture (0·5 ml.) contained 100 mM-sodium cacodylate buffer (pH 7·0), 1 mM-adenosine triphosphate (pH 7·0), 10 mM-$MgCl_2$, 10 mM-KCl, 4 mM-reduced glutathione (pH 7·0), 100 μg of bovine plasma albumin, 1 mM-[^{14}C]glycine (uniformly labeled, 3500 Cts/min/nmole), 20 to 25 A_{260} of tRNA and 0·1 to 1 unit of enzyme activity. (One unit of enzyme activity is equivalent to the formation of 1 nmole of glycyl-tRNA in 10 min at 37°C.) The reaction mixture was incubated for 10 min at 37°C and processed as described by Calendar & Berg (1966). To ensure that initial rates were measured, two different amounts of protein were assayed; and the proportionality of activity with protein concentration was checked. Assays were always done at least two different times on separate batches of cells grown on different days. Duplicate determinations always agreed within $\pm 15\%$.

Serine transhydroxymethylase and tryptophan synthetase activities were assayed as described previously (Folk & Berg, 1970*a*,*b*).

(d) *Isolation of tRNA and measurements of amount of amino acylated tRNA*

The extraction and subsequent treatment of tRNA for measurements of the levels of acylated tRNA *in vivo* were performed as previously described (Folk & Berg, 1970*b*). Briefly, the procedure involves precipitation of cells from growth media with trichloroacetic acid; tRNA is extracted from the precipitate by sonication in phenol and is then partitioned into an aqueous phase buffered at low pH. A portion of the tRNA is treated with periodate to inactivate those tRNA molecules which are not esterified with amino acids, at their 3'OH termini, and then both the oxidized and unoxidized tRNA's are treated with mild alkali to remove esterified amino acids. The remaining specific acceptor capacities of the tRNA's are determined by esterifying them with ^{14}C-labeled amino acids. The specific amino-acid acceptor capacity of the oxidized tRNA divided by the acceptor capacity of the unoxidized tRNA is taken to be the fraction of tRNA esterified *in vivo*. A purified preparation of glycyl-tRNA synthetase, kindly provided by Mr Dennis Ostrem, was used to determine the specific glycine acceptor capacities of the tRNA preparations.

(e) *Bacteria*

Most of the strains described herein are derived from *E. coli* K12 W3110. The isolation and characterization of the *glyS* mutants (BF17, BF51 and BF87) have been previously described (Folk & Berg, 1970*a*,*b*). Derivatives of these strains not previously described are shown in Fig. 1.

Independent isolates of Gly$^+$ revertants were obtained by inoculating approximately 5 Gly$^-$ bacteria into tubes containing minimal media, glucose, other required nutrients and 1 to 5 μg glycine/ml. (this is an insufficient amount to allow full growth of the Gly$^-$ mutants). After each culture was fully grown, clonal isolates (one from each tube) were made by streaking on to minimal agar medium without glycine and these were purified by several restreakings on the same medium.

Xyl$^-$ derivatives were obtained by mutagenizing a full-grown culture with ethyl methane sulfonate (0·1 ml./5 ml. of culture) for 2 hr, then diluting 100-fold into a minimal medium containing glucose and other essential supplements (Eidlic & Neidhardt, 1965); after full growth was achieved, appropriate dilutions were spread on eosin–methylene blue agar containing D-xylose (1%). Xyl$^-$ colonies usually constituted 0·05 to 1·5% of the population.

Thy$^-$ derivatives were obtained after growth in trimethoprim (generously supplied by Dr G. Hitchings, Burroughs Wellcome & Co., Long Beach, N.Y.) as previously described (Folk & Berg, 1970*a*). Mutant *recA56* derivatives of the Thy$^-$ mutants were prepared by mating with JC5088, a *thyA*$^+$*recA56* Hfr, provided by Dr A. J. Clark. Details of this procedure are described by Hill *et al.* (1969).

(f) *Growth of bacteria*

Bacteria were grown in flasks at 37°C on a rotary shaker, unless otherwise noted. Growth was followed by measuring the turbidity at 590 nm. Usually, glycyl-tRNA synthetase assays were performed on cells which had reached stationary phase; other assays were made with cells harvested in late logarithnic growth. Unless otherwise noted, growth conditions intended to stabilize the genetic constitution of the inoculum were used. Revertant strains were not grown in media containing glycine (unless so noted in Results), nor were auxotrophs grown with limiting supplements. Generally, a sample of each culture was streaked on selective media to ensure that its phenotype had not altered during growth.

(g) *Transduction and Hfr matings*

Phage P1kc mediated transduction, and uninterrupted matings with JC5088, were carried out as described by Hill *et al.* (1969). Transductants were clonally purified 3 times on selective media prior to analysis.

(h) *Segregation tests*

Clonally purified isolates were suspended in either L broth containing glycine (1 mg/ml.) or in selective medium (see text), and grown to stationary phase. Appropriate dilutions were plated on supplemented minimal medium (containing the appropriate supplements

so that segregants would not be selected against), or on an indicator medium. If, for example, the clone's requirement for glycine was being examined, colonies from the glycine-supplemented plates were suspended in drops of minimal salts solution, then spot-tested onto selective media. To facilitate multiple testing, a brass plate to which 24 brass nails had been attached in an easily recognizable pattern was used for the spot testing. This method enables the transfer of uniform patches of bacteria so that bacterial growth on the selective medium can be recognized as being due either to growth of a majority of the cells, or to rapid growth of a few revertants in the cell population. In later experiments, Gly^- segregants were detected by spreading samples from the supplemented medium onto minimal medium containing limiting glycine (0·3 μg/ml.). The small Gly^- colonies were easily distinguished from the large colonies formed by Gly^+ bacteria.

3. Results

(a) *Occurrence and properties of Gly⁺ revertants*

Reversion of the various *glyS* mutants from a glycine dependent (Gly^-) to a glycine independent (Gly^+) phenotype occurs with unusual frequency; generally between 10^{-4} and 10^{-5} of cells grown in media containing glycine (1 mg/ml.) will grow without glycine. The doubling times of the revertants as well as their Gly^- progenitors and the wild-type *glyS*⁺ strains in minimal medium and in L broth are summarized in Table 1; the revertants grow nearly as rapidly as the wild type in either the unsupplemented or enriched medium.

TABLE 1

Growth rates of glyS *mutants and their revertants*

Strain	Doubling time	
	Minimal medium	L broth
PB 14a	67 min	30 min
BF 17	>9 hr	64 min
BF 133 (rev. of BF 17)	74 min	32 min
BF 51	>8 hr	67 min
BF 103 (rev. of BF 51)	75 min	30 min
BF 87	>10 hr	70 min
BF 101 (rev. of BF 87)	74 min	35 min

The levels of serine transhydroxymethylase (the enzyme which catalyzes the biosynthesis of glycine from serine) are identical in the *glyS* mutants, the corresponding revertants and in wild-type strains. Thus, reversion to Gly^+ is probably not due to increased synthesis of glycine. In addition, the revertants do not contain higher levels of $tRNA^{Gly}$ nor altered forms of $tRNA^{Gly}$ which could account for normal growth in the absence of exogenously provided glycine.

Whereas the level of esterified $tRNA^{Gly}$ drops to undetectable levels when *glyS* mutants exhaust exogenous glycine from their growth medium (Folk & Berg, 1970*b*), the Gly^+ revertants maintain nearly normal levels of glycyl $tRNA^{Gly}$ while growing in a medium lacking glycine (Table 2). Although the data are not presented here, the efficiency of charging the $tRNA^{Gly}$ species responsible for suppression of the *trpA36* mutation (Carbon, Berg & Yanofsky, 1966; Carbon *et al.*, 1969) is nearly as high as in wild-type strains and between three and eight times higher than the corresponding

TABLE 2

In vivo *levels of glycyl-tRNA^{Gly} in* glyS *mutants and their revertants*

Strain	Growth conditions	% Esterified glycyl-tRNA†
PB14a (*glyS$_H$*)	Exponential; no glycine	81‡
BF87 (*glyS87*)	Exponential; plus glycine	45‡
	After glycine deprivation	2‡
BF101 (rev. *glyS87*)	Exponential; no glycine	57
BF51 (*glyS51*)	Exponential; plus glycine	46‡
	After glycine deprivation	2‡
BF103 (rev. *glyS51*)	Exponential; no glycine	57
BF17 (*glyS17*)	Exponential; plus glycine	33‡
	After glycine deprivation	4‡
BF133 (rev. *glyS17*)	Exponential; no glycine	46
BF202 R-5A (*glyS51/glyS87*)§	Exponential; no glycine	84

† Averages of two or more independent determinations.
‡ Data taken from Folk & Berg (1970*b*).
§ Heterozygote made by transducing *xyl$^+$glyS51* into BF202 R-5.

Gly$^-$ mutant. Both of these results argue that, *in vivo*, there is more efficient charging of tRNA^{Gly} chains in the revertants than in the mutants from which they arose.

(b) *Glycyl-tRNA synthetase levels in the Gly$^+$ revertants*

Reversion from Gly$^-$ to Gly$^+$ is always accompanied by a two- to fourfold increase in glycyl-tRNA synthetase activity, as measured in sonic extracts (Table 3). The increased glycyl-tRNA synthetase activities of the revertants are probably not due to a change in the enzyme *per se* since the K_m values for glycine are virtually identical for the revertant and mutant activities. Moreover, the temperature sensitivity of the enzymes from BF17 and BF87 (see Folk & Berg, 1970*b*) is as pronounced in the revertants as in the mutants. Therefore, it seems that a quantitative change, rather than a qualitative change, in the synthetase proteins accompanies the reversion from Gly$^-$ to Gly$^+$.

(c) *Recovery of the original* glyS *alleles from the revertants*

When any of the Gly$^-$ mutants are used as donors in a phage P1-mediated transduction of a Xyl$^-$Gly$^+$ recipient (PB125:*xyl glyS$_L$*), 60 to 80% of the Xyl$^+$ recombinants are Gly$^-$ (Folk & Berg, 1970*a*). The same result was obtained when the Gly$^+$ revertants were donors: in each case, 60 to 80% of the Xyl$^+$ transductants were Gly$^-$ (Table 4). Several of the Gly$^-$ isolates from each cross were assayed for their glycyl-tRNA synthetase activities, and all were found to have the activities characteristic of the unreverted Gly$^-$ donor. Moreover, when the Gly$^+$ revertants (BF133, BF103 and BF101) were backcrossed to their respective Gly$^-$ progenitors (BF201, BF202 and BF203) the number of Gly$^+$Xyl$^+$ transductants was no more than would have been expected from the rare transduction of the Xyl$^+$ allele into a spontaneously occurring Gly$^+$ revertant in the recipient population (Table 4).

Further proof that reversion from Gly$^-$ to Gly$^+$ is not due to a change in the *glyS* gene follows from the observation that the Gly$^+$ revertants themselves are not stable. Gly$^+$ revertants segregate Gly$^-$ bacteria at high frequencies; even when grown under

TABLE 3

Apparent V_{max} and K_m of glycyl-tRNA synthetase activities in extracts of glyS *mutants and their revertants*

Strain no.	Glycyl tRNA synthetase Activity†	K_m(Gly)‡	V_{max}(apparent)§
PB14a (*glyS*$_H$)	133	0·08	128
BF17 (*glyS17*)	24	1·6	61
BF133 (rev. of BF17)	62	1·2	140
BF51 (*glyS51*)	57	0·6	84
BF103 (rev. of BF51)	182	0·6	213
BF87 (*glyS87*)	1	1·3	3
BF101 (rev. of BF87)	4	1·9	9

All estimates are the average of three or more determinations in which each measurement is within 10% of the mean.

†§ nmoles of glycyl-tRNA formed/10 min/mg protein at 37°C.

‡§ Constants obtained from Lineweaver–Burke plots; K_m is expressed as mM and V_{max} in the above units.

conditions in which the Gly$^-$ segregants cannot multiply, a culture of a Gly$^+$ revertant may contain up to 10% of Gly$^-$ segregants. Such segregants have the growth characteristics and glycyl-tRNA synthetase activity of the *glyS* mutants from which the revertant was derived, indicating that the nature of the reversion does not involve a change in *glyS*.

Both the physiological and enzyme studies argue that reversion to Gly$^+$ is accompanied by an increased amount of glycyl-tRNA synthetase activity *in vivo* and *in vitro*; and yet the mutation responsible for this increase does not alter the structural gene for the enzyme. Although one can imagine a number of different ways in which the amount of the mutant glycyl-tRNA synthetase could be increased, or in which the enzymic activity of the mutant *per se* could be increased, the following genetic evidence suggests that both the increased enzyme activity and the Gly$^+$ phenotype result from an increase in the number of *glyS* gene copies in the revertant. More specifically, we shall try to establish that this involves a duplication (or more generally, amplification) of the chromosomal region encompassing the *glyS* gene.

(d) *Evidence for duplication of the* glyS *region in Gly$^+$ revertants*

(i) *Frequent segregation of Gly$^-$ cells from cultures of the Gly$^+$ revertants*

Gly$^+$ revertants of each of the *glyS* mutants are themselves genetically unstable and segregate Gly$^-$ cells even while growing in a medium which selects against growth of the Gly$^-$ cells (minimal medium). In some cases cultures of the Gly$^+$ revertants contain 5 to 10% of the population as Gly$^-$ cells while others have fewer segregants (0·1 to 1%) during growth under the same conditions. In every case, sonic extracts made from the Gly$^-$ cells recovered by segregation from their Gly$^+$ progenitors had glycyl-tRNA synthetase activities characteristic of the original *glyS* mutant. Gly$^-$ segregants derived from the Gly$^+$ revertants are indistinguishable from the original Gly$^-$ isolates in the frequency with which they regenerate the Gly$^+$ phenotype. Therefore, the mutation causing loss of the Gly$^+$ phenotype restores the mutant enzyme level and the propensity for regenerating the unstable Gly$^+$ phenotype.

Similar genetic instability has already been observed in strains containing tandemly repeated *lac* genes (Horiuchi *et al.*, 1963) and heterozygous suppressor genes (Hill *et al.*, 1969; Soll & Berg, 1969; Russell *et al.*, 1970); segregation was prevented when the cell was rendered recombination deficient. Accordingly, two Gly$^+$ revertants were made *recA56* (see Materials and Methods); whereas cultures of the *rec*$^+$ revertants contained between 3 and 20% segregants when grown in rich media, the *recA56* derivatives contained less than 0·1% (0/1584 and 0/2750) segregants. Thus, the frequent loss of the gene conferring prototrophy probably occurs by a process requiring genetic recombination.

(ii) *Construction of strains heterozygous at the* glyS *allele*

If, indeed, reversion from the Gly$^-$ phenotype (due to *glyS* mutation) to Gly$^+$ is due to an increase in the number of *glyS* gene copies, it should be possible to replace one of these copies with another *glyS* allele; i.e. to make the cell heterozygous for *glyS*. Moreover, if other genes near the *glyS* region are also increased in number by the same event, these may also be changed from their homozygous to a heterozygous state.

The construction of one such partially heterozygous strain was carried out as follows (see Table 5). A Gly$^-$Xyl$^-$ (*glyS51 xyl*) mutant (A) was reverted to Gly$^+$ (B). The prototroph was still Xyl$^-$ and, as mentioned in an earlier section, this Gly$^+$ revertant contained about two to three times the glycyl-tRNA synthetase activity of the mutant; these Gly$^+$ cells segregated Gly$^-$ cells (B-1) which had the enzyme activity characteristic of BF51. The Gly$^+$ revertant was infected with P1 phage grown on a Gly$^-$Xyl$^+$ (*glyS87*) strain and the Xyl$^+$ transductants were selected on medium containing xylose and glycine and then scored for their glycine requirement. Nearly equal numbers of Gly$^-$Xyl$^+$ (D) and Gly$^+$Xyl$^+$ (E) clones were found. The Gly$^-$Xyl$^+$ transductants (D) generally had glycyl-tRNA synthetase activities corresponding to the Xyl$^+$ donor strain (in this case of *glyS87* strains); the origin of these transductants will be commented upon in the Discussion in connection with the genetic structure of

TABLE 4

Transduction of mutant glyS *allele from* glyS *mutants and their revertants*

Donor	Recipient	Per cent of Gly$^-$ among Xyl$^+$ transductants
BF17 (*glyS17*)	PB125 (*xyl glyS*)	67
	BF200 (*xyl glyS17*)	<0·01
BF51 (*glyS51*)	PB125	60
	BF201 (*xyl glyS51*)	<0·02
BF87 (*glyS87*)	PB125	71
	BF202 (*xyl glyS87*)	<0·08
BF133 (rev. of *glyS17*)	PB125	66
	BF200 (*xyl glyS17*)	<0·01
BF103 (rev. of *glyS51*)	PB125	78
	BF201 (*xyl glyS51*)	<0·01
BF101 (rev. of *glyS87*)	PB125	74
	BF202 (*xyl glyS87*)	<0·01

The cotransduction frequency of *glyS* and *xyl* has ranged between 60 and 80% in many separate measurements.

TABLE 5

Glycyl-tRNA synthetase activities of glyS *mutant, revertant and partially heterozygous strains*

Phenotype and inferred genotype of strains referred to in text	Reference letter	Comment on strain origin	Glycyl-tRNA synthetase activity (units/mg protein)
Gly$^-$Xyl$^-$ (*glyS51 xyl*)	A	Original mutant	50–70
Gly$^+$Xyl$^-$ (*glyS51 xyl/glyS51 xyl*)	B	Reversion to Gly$^+$	180–200
Gly$^-$Xyl$^-$ (*glyS51 xyl*)	B-1	Gly$^-$ segregant of B	50–70
P1 (*glyS87*)	C		1–2
Gly$^-$Xyl$^+$ (*glyS87*)	D	Two classes of Xyl$^+$ transductants recovered from infection of B by C	1–2
Gly$^+$Xyl$^+$ (*glyS51 xyl/glyS87 xyl*$^+$)	E		85–95 (4†) 161 (1)
Gly$^-$Xyl$^+$ (*glyS87*)	E-1	Gly$^-$ segregants from E	1 (5)
Gly$^-$Xyl$^-$ (*glyS51 xyl*)	E-2		60 (2)

† Number in parenthesis indicates the number of clones with indicated enzyme activity.

the Gly$^+$ revertants. The Gly$^+$Xyl$^+$ transductants (E) usually had enzyme activities markedly lower than the Gly$^+$Xyl$^-$ strain used as recipient for the P1 transduction; in this case, then, although transduction of the Xyl$^+$ character did not alter the Gly phenotype, the level of glycyl-tRNA synthetase activity was approximately halved (Table 5). The Gly$^+$Xyl$^+$ clones readily yielded two kinds of Gly$^-$ segregants; Gly$^-$Xyl$^+$ (E-1) having synthetase activities identical to the Xyl$^+$ donor (*glyS87*), and Gly$^-$Xyl$^-$ (E-2) having enzyme activities identical to the recipient (*glyS51*). Quite clearly, the transduction generated a Gly$^+$ phenotype in cells containing both the *glyS51* and *glyS87* alleles; in this case, segregation of the two alleles yields Gly$^-$ cells containing enzymes characteristic of *glyS51* or *glyS87* mutants.

The same type of experiment has been done starting with a Gly$^-$Xyl$^-$ (*glyS87 xyl*) strain and introducing the *glyS51* allele into the Gly$^+$Xyl$^-$ revertant; in this case, the results were analogous to those described above except that glycyl-tRNA synthetase activity characteristic of *glyS51* segregated with Xyl$^+$ and the activity associated with *glyS87* with Xyl$^-$. At least 12 other independently isolated revertants have been examined in a more limited way and in every respect they were quite similar to the more extensively characterized ones above.

Both experiments also show that introduction of Xyl$^+$ by P1 transduction also generates Xyl$^+$ clones which segregate Xyl$^+$ and Xyl$^-$ cells at high frequency. This is quite different from the result one finds when Xyl$^+$ is transduced into the standard Xyl$^-$, for example, the original Gly$^-$Xyl$^-$ strains shown in Figure 1; in the usual case, stable Xyl$^+$ transductants are recovered almost exclusively.

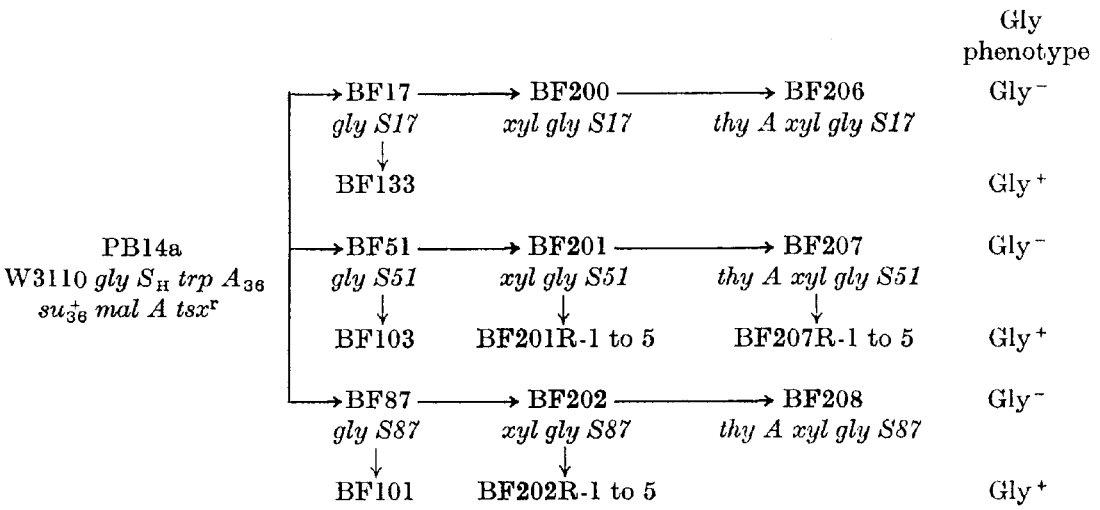

FIG. 1. Bacterial strains. Mutations were introduced as described in Materials and Methods. Strains pointed by a horizontal arrow are Gly⁻; those pointed by a vertical arrow are Gly⁺ by spontaneous reversion.

(iii) *Extent of the chromosome involved in the duplication*

If, as suggested above, reversion of the Gly⁻ phenotype to Gly⁺ results from an increased number of copies of the *glyS* locus, one may inquire as to whether other nearby genes are also included in the duplicated segment. To estimate the extent of the duplication, we constructed other partial heterozygotes. Five independent Gly⁺ revertants were isolated from BF201 (*glyS51 xyl glpD*⁺ *malA strA*⁺) and from BF202 (*glyS87 xyl glpD*⁺ *malA strA*⁺). The first set of five revertants (from BF201) was transduced to Xyl⁺ with P1 phage grown on the mutant BF87 (*glyS87 xyl*⁺) and the other five revertants (from BF202) were also transduced to Xyl⁺, but with P1 phage grown on BF51 (*glyS51 xyl*⁺); in each transduction, representative Gly⁺Xyl⁺ clones were purified and tested for segregation of the Gly and Xyl phenotypes. Transductants from eight of the original ten revertants segregated Gly⁻Xyl⁻ cells indicating that in these eight revertants, at least two *xyl* alleles had very likely been present prior to the transduction.

Similarly, P1 phage transduction to Mal⁺ of each of these ten revertants yielded unstable Mal⁺ clones from three of the revertants. In these three cases, almost all of the Mal⁻ segregants were also Gly⁻; thus, these three revertants probably contained at least two *malA* genes prior to the transduction.

Of the three Gly⁺ revertant strains which contained multiple *malA* genes, two also contained multiple *xyl* genes while the other probably did not. In these two, therefore, the duplication of genetic material extended from *glyS* to *malA* (approximately 4% of the *E. coli* chromosome; see Fig. 2). The fact that one of three of the amplifications did not also include the *xyl* locus suggests that the closely linked *xyl* and *glyS* genes have the following order with respect to *malA*: *xyl-glyS-malA*. The order could not be *xyl-malA-glyS* because *glyS* is cotransducible with *xyl* but not with *malA*.

If reversion to Gly⁺ has generated duplicate copies of the *glyS* and *malA* genes, loci located between them would also be increased in number. Accordingly, we determined whether the structural gene for L-α-glycerophosphate dehydrogenase (Cozzarelli, Freedberg & Linn, 1968), *glpD* (see Fig. 2), is duplicated in some of the Gly⁺ revertants. Ordinarily, about 80% of the Mal⁺ transductants produced from a *malA*⁺

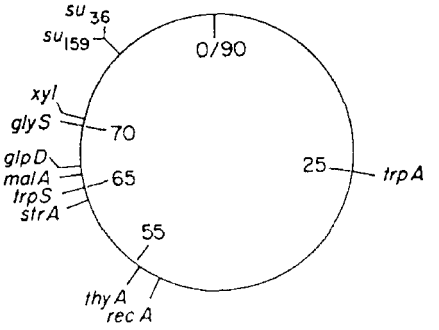

FIG. 2. Genetic map of *E. coli*. Genetic distances and map order are taken from Taylor & Trotter (1967).

glpD donor (strain 95 (*malA*$^+$*glpD*) was provided by Dr E. C. C. Linn) are also Glp$^-$. Yet when the three Gly$^+$ revertants which contained more than one *malA* gene (see above) were used as recipients for the transduction, no Mal$^+$Glp$^-$ transductants were found (Table 6); if the revertants contained only one copy of *glpD* and the same genetic linkage of *malA* and *glpD* still existed in the revertants, one would have expected to find Mal$^+$Glp$^-$ recombinants; if there was more than one copy, the wild-type *glpD*$^+$ allele would be dominant and thereby mask the cotransduction event. Direct evidence that nearly the expected number of Mal$^+$ transductants were also made heterozygous for *glpD* is the fact that from better than 50% of the Mal$^+$Glp$^+$ transductants we could recover Glp$^-$ clones amongst the Gly$^-$ segregants.

In none of the revertant strains tested does the duplicated region extend through the nearby *trpS* or *strA* genes (see Fig. 2); these strains have exactly the same amount of tryptophanyl-tRNA synthetase activity (specified by the *trpS* locus) as a haploid strain, and all three of the strains that contained multiple *malA* genes could be trans-

TABLE 6

Evidence for more than one glpD *allele in cells containing multiple copies of the* GlyS *and* malA *alleles*

Recipient for transduction with P1 (*glpD malA*$^+$)†	Glp$^-$Mal$^+$ / Mal$^+$	Cotransduction frequency %	Segregants found from Mal$^+$ transductants Glp$^-$	Mal$^-$
BF201 (*glyS51 glpD*$^+$ *malA*)	24/36	66	No	No
BF202 (*glyS87 glpD*$^+$ *malA*)	27/36	75	No	No
BF201 rev. 1 (*glyS51 glpD*$^+$ *malA*/*glyS51 glpD*$^+$ *malA*)	0/48	<3	Yes	Yes
BF201 rev. 3 (*glyS51 glpD*$^+$ *malA*/*glyS51 glpD*$^+$ *malA*)	0/35	<3	Yes	Yes
BF202 rev. 5 (*glyS87 glpD*$^+$ *malA*/*glyS87 glpD*$^+$ *malA*)	0/36	<3	Yes	Yes

† The designations in parenthesis indicate the putative genotypes.

duced to *str*^{R} at the same frequency as a haploid *str*^{S} strain. Had the amplification included *str*^{S}, it is unlikely that any *str*^{R} transductants would have been obtained, for *str*^{S} is dominant to *str*^{R} in diploids (Lederberg, 1951; Sparling, Modolell, Takeda & Davis, 1968).

(iv) *Segregation of genetic markers included in the duplicated segment*

Using a multiply-marked heterozygous strain, the different types of recombinational segregation events which can occur in the duplicated segment could be examined. To do this, the revertant derived from BF201 (*xyl glyS51 glpD*$^+$ *malA*), and shown to have undergone duplication of the markers *xyl glyS* and *malA*, was made heterozygous for *xyl*, *glyS*, *glpD* and *malA*. This was accomplished by successive transductions in which *xyl*$^+$*glyS87* was introduced first, followed by transduction of *glpD malA*$^+$. Assuming that the Gly$^+$ revertant of BF201 (*xyl glyS51 glpD*$^+$ *malA*) had a duplication of the following type *xyl glyS51 glpD*$^+$ *malA* : *xyl glyS51 glpD*$^+$ *malA*†, four types of derivatives with the phenotype Xyl$^+$Gly$^+$Glp$^+$Mal$^+$ could be anticipated from the two successive transductions mentioned above. These are:

(a) *xyl*$^+$ *glyS87 glpD malA*$^+$: *xyl glyS51 glpD*$^+$ *malA*
(b) *xyl glyS51 glpD*$^+$ *malA* : *xyl*$^+$ *glyS87 glpD malA*$^+$
(c) *xyl*$^+$ *glyS87 glpD*$^+$ *malA* : *xyl glyS51 glpD malA*$^+$
(d) *xyl glyS51 glpD malA*$^+$: *xyl*$^+$ *glyS87 glpD*$^+$ *malA*.

Although these putative heterozygotes cannot be distinguished phenotypically, the segregation data presented in Table 7 and discussed below favor configurations (c) or (d).

Consider first, segregants selected for their inability to utilize xylose and then scored as to their Gly, Glp and Mal phenotypes (Table 7). Nearly 75% were Gly$^-$, Glp$^-$ and Mal$^+$, suggesting a loss of the left segment of genes in (c), or loss of the right segment of genes in (d). Essentially all of the Glp$^-$ segregants had also lost the markers proposed for the left segment of (c), or for the right segment of (d). Moreover, of the Mal$^-$ segregants, 70% had lost the markers attributed to the right segment of (c) or to the left segment of (d). It seems, therefore, that the predominant mode of segregation of genes included in the amplification is probably by elimination of an entire piece of genetic information. If the genetic structure were a tandem sequence of homologous sets of genes, a single recombinational event leading to an excision of one-half of the repetition could account for this form of segregation; similarly, loss of an episome carrying the appropriate set of markers would also suffice.

There are also other but less frequent classes of segregants (Table 7). All of them require multiple recombinational events and can be accounted for by the structure suggested for (c) or (d) above. However, since the analysis is relatively limited at this stage, it would be premature to draw too many conclusions as to the detailed fine structure of this genetic region.

† Although we have used the term duplication, our data do not yet distinguish a tandem duplication from multiple tandem repeats of a gene sequence or even two representations of a gene sequence in which one is in the chromosome and one or more are on episomal elements. Nevertheless, we have used the term duplication to mean the process leading to the increased number of copies of the genetic region in question. The symbol : is used to designate the "repeat point", or point at which genetic repetition begins, although there might be other genes after *malA* or before *xyl* included in the repeated sequence.

TABLE 7

Segregation patterns from a multiply-marked heterozygote having the phenotype Xyl$^+$ Gly$^+$ Glp$^+$ Mal$^+$

Selected phenotype	Scored phenotype Xyl	Gly	Glp	Mal	Frequency
Xyl$^-$	−	−	−	+	34/46
	−	+	+	+	4/46
	−	−	+	−	3/46
	−	−	−	−	2/46
	−	−	+	+	2/46
	−	+	−	+	1/46
Glp$^-$	−	−	−	+	21/21
Mal$^-$	+	−	+	−	34/48
	−	−	+	−	13/48
	+	−	−	−	1/48
Xyl$^-$Mal$^-$	−	−	+	−	21/23
	−	−	−	−	2/23

Segregants from the heterozygous strain described in the text were detected on indicator media (selected phenotype) and after purification they were scored for other phenotypic markers on appropriate selective media or indicator media (scored phenotype).

(e) *Other types of revertants*

Are there other types of revertants than those which become Gly$^+$ as a result of an increase in the number of gene copies of *glyS*? In nine of ten independent revertants of BF17 (*glyS17*), we observed about a threefold increase of glycyl-tRNA synthetase activity in sonic extracts; in each of these nine revertants, it could be shown by transduction that the structural gene, *glyS17*, remained unchanged. One out of the ten had a glycyl-tRNA synthetase activity identical to its parent; although genetic analysis showed that the site of reversion was outside the *glyS17* structural gene, it differs from the other revertants described in this paper in that it is genetically stable. In general then, the predominant class of revertants of the *glyS* mutants is one that undergoes an event producing an increased number of the mutant *glyS* gene.

4. Discussion

A relatively frequent ($>10^{-5}$) reversion of the Gly$^-$ phenotype to Gly$^+$ amongst several different classes of *glyS* mutants without, at the same time, any alteration in the *glyS* gene was an unexpected finding. The revertants appear to be similar to wild-type *glyS*$^+$ strains in most respects. Whereas the original *glyS* mutants fail to grow on minimal medium unless it is supplemented with glycine, the revertants have normal generation times in media lacking glycine; even in a rich medium (Table 1), the mutants grow at half the rate of the wild type but under these conditions revertant and wild-type cultures have indistinguishable growth rates. The *glyS* mutants esterify tRNA^{Gly} *in vivo* only when the medium contains glycine, whereas the revertants maintain nearly normal intracellular levels of glycyl-tRNA in the absence of glycine. The revertants still contain the same mutationally altered structural *glyS* gene and produce the mutant form of the glycyl-tRNA synthetase. But there is a two- to fourfold increase in the synthetase activity of extracts prepared from revertant

cells compared to their progenitors, suggesting that the revertant produces more of the defective enzyme.

There are, in principle, several different ways to account for the elevated level of mutant glycyl-tRNA synthetase; our data indicate, however, that an increase in the number of *glyS* gene copies probably accounts for the increased enzyme activities measured *in vitro*, the increased intracellular levels of the glycyl-tRNA and, therefore, the prototrophic phenotype. The most convincing evidence that the revertants contain more than one copy of the *glyS* allele is the observation that the putative *glyS51*/*glyS51* homozygous partial diploid is readily converted to the heterozygous equivalent by introduction of *glyS87*; such heterozygotes (which are phenotypically Gly^+) segregate the two expected classes of Gly^- auxotrophs, *glyS51* and *glyS87*. Moreover, each of the three possible putative homozygous partial diploid revertants can be converted to segregating heterozygotes by introduction of one of the other *glyS* alleles. The relatively high frequency with which Gly^- cells arise from the Gly^+ revertants, as well as from their heterozygous derivatives, and the fact that linked genes are usually lost, concomitantly argues for the existence in these cells of repeated segments of the *glyS* region in the genome; since the segregation of Gly^- cells from the Gly^+ revertants is eliminated by making the cells recombination deficient (*recA*), we suspect that a recombinational event is involved in loss of the partial diploid state.

The genetic evidence only allows one to say that there is more than one copy of the *glyS* gene in the revertants; conceivably some of the revertants could have more than two *glyS* copies. To test more directly that two copies of the mutant *glyS* allele are sufficient to establish prototrophy, we attempted to construct merodiploids in which another copy of the *glyS xyl* region could be carried on an F′ element. However, our attempts to establish such F′ merodiploids have not yet been successful.

Our present data do not allow us to decide whether the multiple copies of the *glyS xyl* region exist in tandem within the host chromosome or whether one or more exists on an autonomously replicating plasmid. The rate of spontaneous segregation is so high that acridine curing experiments (Hirota, 1960) are useless. The cells are phenotypically F^- before and after reversion so that class of episome is unlikely. Whether there are yet unidentified plasmid elements in our strain into which the duplicate segment could be integrated remains to be determined. The segregation pattern obtained from a multiply-marked heterozygote (Table 7) is quite easily rationalized by single (the most frequent) or multiple recombinational events within a tandemly repeated sequence (Campbell, 1963); to account for the same data with an episome model requires more complicated interchanges of markers between the chromosome and episome, followed by loss of the episome; moreover, to account for the Gly^+ phenotype of certain segregants one would have to explain how diploidy for the *glyS* locus is maintained while at the same time episomal markers are eliminated.

As mentioned previously (see Results, section (d)(ii) and Table 4) transduction of *xyl*$^+$*glyS* into a Gly^+ revertant yields approximately equal numbers of Xyl^+Gly^+ and Xyl^+Gly^- recombinants. The frequency of Xyl^+Gly^- cells is much too high to be explained by the transduction of the small number of Gly^- in the recipient population to Xyl^+. Rather, this result suggests that the transduction event itself generates the Gly^- cells. There is no simple way to explain this if the duplicated region is extrachromosomal. However, if the duplicated region is integrated into the chromosome, the results may be explained by postulating a recombinational event in which the

region of DNA near *xyl* on the donor DNA pairs with a homologous region on one of the copies of the duplicated region in the recipient chromosome, and the region of DNA near *glyS* on the donor DNA pairs with a homologous region on *a different* copy of the duplicated region in the recipient chromosome. Recombinational events at the two paired regions leading to integration of the donor DNA would simultaneously lead to loss of the duplicated region between the two genes. The resulting recombinant would be Xyl^+Gly^-, with the *glyS* allele derived from the donor DNA, as is observed.

We feel that the majority of these results are consistent with the interpretation that the multiple copies of *glyS* and the linked genes are linearly integrated into the *E. coli* chromosome, but additional work will be needed to substantiate this.

Our experiments show clearly that duplication (amplification) of the *glyS* gene occurs relatively frequently ($>10^{-5}$) suggesting that only a single event is necessary. In this event, variable portions of the chromosome adjacent to *glyS* are included in the duplication; the largest region encompasses *xyl* and *malA*, about 4·5 minutes of the chromosomal map (Taylor & Trotter, 1967), and although we have looked for more extensive duplications (e.g. to include *glyS* and *strA*), we have not yet found them. The nature of the event which causes the duplication is obscure and needs further study.

The frequency and chromosomal distribution of gene duplications are difficult to determine. Quite clearly, duplication of genetic material can be selected for and maintained only when it is essential for cell survival and growth. Horiuchi *et al.* (1963) selected for strains containing multiple chromosomal copies of the *lac* genes by growing bacteria for long periods of time at limiting concentrations of lactose in the chemostat; since multiple sets of lac genes were tranduced by a single P1 phage particle, the extent of the duplication was probably small. As one might expect, the extra genes were lost frequently (10^{-2}) by segregation and considerable genetic recombination between duplicated segments occurred. Hill *et al.* (1969) and Carbon *et al.* (1969) showed that to maintain a missense suppressor mutation (one causing a change in a $tRNA^{Gly}$ species) the cell also carries in tandem the wild-type allele of the suppressor locus. Russell *et al.* (1970) detected duplicate, tandemly situated copies of the structural gene for $tRNA^{Tyr}$. Genetic duplications have been observed after Hfr $\times$ F^- matings (Curtiss, 1964; Campbell, 1965), and phage P1 has been implicated in the formation of partial heterozygotes which are quite unstable (Lennox, 1955; Luria, Adams & Ting, 1960; Soll & Berg, 1969). Also, Ames, Hartman & Jacob (1963) have characterized a mutant of *Salmonella typhimurium* which has a region of the *his* operon duplicated. Before a complete appreciation of the importance of such chromosomal changes can be achieved, additional studies with systems in which gene duplication can easily be detected and analyzed are needed.

We can only speculate now on why duplication of the mutant *glyS* locus accounts for the loss of auxotrophy and nearly normal rates of $tRNA^{Gly}$ charging *in vivo*. It seems unlikely that the reversion of *glyS17* and particularly *glyS87* can be explained by the increases in glycyl-tRNA synthetase activity we measure *in vitro* (see Table 3). One possible explanation for how multiple copies of the mutant *glyS* gene can account for reversion of the mutant phenotype emerges from recent findings by Ostrem & Berg (1970) of the structure of glycyl-tRNA synthetase. They find that the enzyme is a tetrameric protein containing two subunits of molecular weight 33,000 (α) and two subunits of molecular weight 80,000 (β); since the molecular weight of the native enzyme is 235,000, the protein probably has the structure $\alpha_2\beta_2$. Both subunits are

coded for by the *glyS* locus and, indeed, some of the mutants have defective α subunits while others have altered β subunits. The isolated subunits are enzymically inactive but the active oligomeric structure can be assembled from the subunits. Given a simple equilibrium of the form $\alpha_2\beta_2 \rightleftharpoons 2\alpha + 2\beta$, it is clear that, depending upon the constant for association of the subunits, the concentration of α and β subunits can have a profound influence on the amount of the $\alpha_2\beta_2$ species. If in the mutants, association of the subunits is impaired and there exists a pool of free subunits, a doubling of subunit concentration due to higher gene dosage would have a multiplicative effect on the level of the $\alpha_2\beta_2$ oligomer: how much of an effect (between 2 and 16 times) would depend upon the association constant for the mutant $\alpha_2\beta_2$ complexes. A multiplicative effect of subunit concentration on the amount of active oligomer has already been observed in the case of the *lac* repressor protein by Sadler & Novick (1965); they found a disproportionate increase in the level of *lac* repressor (between 16 and 34 times) for a 2·0- to 2·5-fold increase in the number of *lac* repressor genes and they predicted that the active form of the *lac* repressor would be a tetramer, a conclusion which is now generally accepted.

Measurements of the glycyl-tRNA synthetase activities in extracts of the revertants and their corresponding mutants shows only a two- to fourfold increase. Whether this is the extent of the increased activity *in vivo* is not known. We have reason to believe that the mutant $\alpha_2\beta_2$ complexes dissociate more readily than does the wild-type enzyme but we lack detailed information of the parameters characterizing that equilibrium. We hope to study the monomer–oligomer equilibrium with purified subunits from wild-type and mutant glycyl-tRNA synthetases.

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